The influence of commensal and pathogenic gut microbiota on prion disease pathogenesis

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Summary

Prion diseases are a unique group of transmissible, chronic, neurodegenerative disorders. Following peripheral exposure (eg: oral), prions often accumulate first within the secondary lymphoid tissues before they infect the central nervous system (CNS). Prion replication within secondary lymphoid tissues is crucial for the efficient spread of disease to the CNS. Once within the CNS, the responses of innate immune cells within it can have a significant influence on neurodegeneration and disease progression. Recently there have been substantial advances in our understanding of how cross-talk between the host and the vast community of commensal microorganisms present at barrier surfaces such as the gut, influence the development and regulation of the host's immune system. These effects are evident not only in the mucosal immune system in the gut, but also in the CNS. The actions of this microbial community (the microbiota) have many important beneficial effects on host health, from metabolism of nutrients and regulation of host development, to protection from pathogen infection. However, the microbiota can also have detrimental effects in some circumstances. In this review we discuss the many and varied interactions between prions, the host and the gut microbiota. Particular emphasis is given to the ways by which changes to the composition of the commensal gut microbiota or congruent pathogen infection may influence prion disease pathogenesis and/or disease susceptibility. Understanding how these factors influence prion pathogenesis and disease susceptibility is important for assessing the risk to infection and the design of novel opportunities for therapeutic intervention.

Key words: Prions; transmissible spongiform encephalopathy; microbiota; intestine; central nervous system; co-infection; microglia.

Abbreviations: BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; CNS, central nervous system; CWD, chronic wasting disease; FDC, follicular dendritic cell; GALT, gut-associated lymphoid tissue; ILF, isolated lymphoid follicle; MNP, mononuclear phagocytes; PrP, prion protein; SCFA, short chain fatty acids;

INTRODUCTION

Prion diseases, or transmissible spongiform encephalopathies, are sub-acute neurodegenerative diseases which affect humans and certain domestic and free-ranging animal species (Table 1). Prion diseases are characterized by the presence of aggregations of PrP^{Sc}, an abnormally folded isoform of the host-encoded cellular prion protein (PrP^{C}), in affected tissues (Bolton *et al.*, 1982, Prusiner *et al.*, 1982). Prions are unique amongst infectious agents in that they appear to lack nucleic acid, comprising solely of the PrP^{Sc} protein (Legname *et al.*, 2004, Wang *et al.*, 2010). The accumulation of PrP^{Sc} in the central nervous system (CNS) of prion-infected hosts is accompanied by reactive microglial and astroglial responses, and significant levels of neurodegeneration. A diverse range of cellular functions have been ascribed to the cellular PrP^{C} protein including maintenance of circadian rhythms (Tobler *et al.*, 1996), signal transduction (Spielhaupter & Schatzl, 2001), seizure sensitivity (Walz *et al.*, 1999), cognition (Coitinho *et al.*, 2003), maintenance of peripheral myelin (Bremer *et al.*, 2010) and phagocytosis of apoptotic cells (de Almeida *et al.*, 2005). However, the precise physiological role remains controversial as some, with the exception of peripheral myelin maintenance, have been since shown to be due to consequences of flanking gene issues or spurious overexpression of Doppel protein in certain PrP-deficient mouse lines (Nuvolone *et al.*, 2016, Nuvolone *et al.*, 2013, Steele *et al.*, 2007).

Some prion diseases have an idiopathic aetiology, apparently arising spontaneously within the CNS (eg: sporadic Creutzfeldt-Jakob disease; CJD). Others such as Gerstmann-Straussler-Scheinker syndrome are associated with polymorphisms within the *PRNP* gene (which encodes PrP^{C}), which may predispose PrP^{C} to abnormally fold into the pathogenic isoform, whereas other

polymorphisms may protect against disease transmission (Asante *et al.*, 2015). Many other prion diseases are acquired, such as following oral consumption of prion-contaminated food. These include natural sheep scrapie, bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) in cervid species such as deer and elk, and variant Creutzfeldt-Jakob disease (vCJD) in humans (Table 1).

The gut-associated lymphoid tissues (GALT) are a group of multi-follicular structures including the tonsils, Peyer's patches, appendix, colonic and caecal patches, as well as individual follicular structures termed isolated lymphoid follicles (ILF). GALT occur throughout the gastrointestinal tract and along with the mesenteric lymph nodes help maintain homeostasis within the gut and protect the host from pathogen infection. After oral exposure the replication of prions within Peyer's patches in the small intestine is crucial for their efficient transmission to the CNS (termed *neuroinvasion*) (Donaldson *et al.*, 2015b). Within them, the prions replicate upon the surfaces of stromal-derived follicular dendritic cells (FDC) located within the B-cell follicles (Mabbott *et al.*, 2000, Mabbott *et al.*, 2003, McCulloch *et al.*, 2011, Montrasio *et al.*, 2000). The prions then infect neighbouring enteric nerves, and spread from them via the sympathetic and parasympathetic nervous systems to the CNS where they cause neurodegeneration and the eventual death of the host (Beekes & McBride, 2000, Glatzel *et al.*, 2001, Kujala *et al.*, 2011, McBride *et al.*, 2001).

The mammalian gastrointestinal tract is home to a vast community of commensal microorganisms, termed the microbiota (Sommer & Backhead, 2013), and the colon of a typical 70 kg human male is estimated to harbour approximately 3.9×10^{13} bacteria (Sender *et al.*, 2015). The commensal gut microbiota provides many beneficial effects on host health including metabolizing nutrients (Russell *et*

al., 2013), influencing the development and regulation of the immune system (Furusawa *et al.*, 2013, Hooper *et al.*, 2012), as well as outcompeting pathogens for nutrients or habitats (Kamada *et al.*, 2012). Rapid advances in high-throughput sequencing technology have helped to gain a greater understanding of the true abundance and complexity of the commensal microbiota (Claesson & O'Toole, 2010). This in-turn has helped reveal how factors such as diet, host genotype (Carmody *et al.*, 2015), antibiotic use (Gibson *et al.*, 2015), pathogen infection (Holm *et al.*, 2015), host age and even the residential environment (Claesson *et al.*, 2012) can affect both the composition of the gut microbiota and host health.

Disturbances to the abundance and complexity of the microbiota can dramatically affect immune regulation and function, and are contributory factors in the development of some inflammatory and autoimmune diseases (Frank *et al.*, 2007). These influences extend far beyond the GALT which have a close physical relationship with the microbiota. Remarkably, the gut microbiota can also affect the development of the CNS. The gut microbiota of mice can influence the development of certain neuronal circuits such as those involved in anxiety-like behaviour (Sudo *et al.*, 2004), and constitutively controls the maturation and function of microglia within the CNS (Erny *et al.*, 2015) (discussed below). Furthermore, diet-induced increases in bacteria from the order Clostridiales and a decrease in Bacteroidales, are associated with poor cognitive flexibility (Magnusson *et al.*, 2015).

In this review we discuss how imbalances to the composition or abundance of the host commensal microbiota, or congruent pathogen infection, may influence prion disease pathogenesis and susceptibility.

Effect of changes to the abundance and complexity of the gut microbiota on prion disease

Effects on oral prion disease pathogenesis: Cross-talk between the host immune system and the microbiota is critical for the development of the immune system, especially the GALT, which can in-turn regulate the microbiota. At the time of writing this review there were no published studies which had directly addressed the influence of the commensal gut microbiota on oral prion disease pathogenesis. Therefore, discussed below are studies which help to shed light on the effects that disturbances to the commensal gut microbiota may have.

A dramatic reduction in the microbiota at the time of oral prion exposure could potentially impede disease pathogenesis. One pronounced effect that the gut microbiota has on the host is the dynamic regulation of ILF development in the intestine (Donaldson *et al.*, 2015a, Hamada *et al.*, 2002) (Fig. 1). ILF are inductive sites for immunoglobulin-A production and can be classified as either immature ILF (primary B-cell follicles), or mature ILF containing a single organized germinal centre, with a network of FDC and an overlying epithelium containing M cells (Donaldson *et al.*, 2015a, Glaysher & Mabbott, 2007a, Lorenz *et al.*, 2003). ILF abundance is reduced in the small intestines of germ-free mice (which lack a gut microbiota), whereas ILF development is induced upon microbial colonisation (Donaldson *et al.*, 2015a). Our data show that ILF are important sites of prion accumulation and neuroinvasion in the small intestine (Donaldson *et al.*, 2015b, Glaysher & Mabbott, 2007b). Furthermore, oral prion disease susceptibility is reduced in the specific absence of GALT (ILF or Peyer's patches) in the small intestine (Donaldson *et al.*, 2015b, Glaysher & Mabbott, 2007b, Horiuchi *et al.*, 2006, Prinz *et al.*, 2003). These observations suggest that a microbiota-induced

reduction in ILF density in the small intestine could impede disease pathogenesis by reducing the available sites of prion uptake, replication and neuroinvasion in the gut.

Unlike in the small intestine where the microbiota promotes ILF development, the microbiota inhibits ILF development in the large intestine (Fig. 1) (Donaldson *et al.*, 2015a). However, our experiments in mice show that large intestinal GALT are not important sites of prion neuroinvasion after oral exposure (Donaldson *et al.*, 2015a). Furthermore, an increased density of ILF specifically in the large intestine does not influence oral prion disease pathogenesis or susceptibility (Donaldson *et al.*, 2015b). Large intestinal GALT are also not considered to be important early sites of prion accumulation in humans exposed to vCJD (Hill *et al.*, 1999, Peden *et al.*, 2004), sheep with natural scrapie and BSE (van Keulen *et al.*, 2008a, Van Keulen *et al.*, 2008b) and cervids with CWD (Spraker *et al.*, 2006, Spraker *et al.*, 2009). The early accumulation of prions in the ileal Peyer's patches of cattle experimentally exposed to BSE has also been described (Katz *et al.*, 2012).

Pathogen infection can also disturb the composition of the commensal gut microbiota. For example, a chronic infection of mice with the large intestinal nematode *Trichuris muris* decreases bacterial diversity, and increases the relative abundance of *Lactobacillaceae* (Holm *et al.*, 2015) (Fig. 2). However, congruent infection with *T. muris* at the time of oral prion exposure does not influence disease pathogenesis (Donaldson *et al.*, 2015b) (Table 2). Therefore, unlike in the small intestine where microbiota-mediated changes to GALT status may influence disease susceptibility, these data suggest that alterations to the abundance or diversity of the commensal microbiota in the large intestine at the time of oral prion

exposure are unlikely to have a significant impact on disease pathogenesis and susceptibility. However, this issue is not straightforward as a dramatically reduced gut microbiota has other important effects on the host. Germ-free mice display substantially enlarged caeca, reduced spleen size (Reikvam *et al.*, 2011) and altered gastrointestinal motility and transit time (Kashyap *et al.*, 2013). Each of these could potentially influence oral prion disease pathogenesis.

Effects on CNS prion disease: A bidirectional communication system, termed the gut-brain axis, integrates neural, hormonal and immunological signalling between these two distantly situated tissues. This enables the brain to influence a variety of physiological activities in gut including motility and secretion, and the actions of the mucosal immune system (Collins *et al.*, 2012). Conversely, gut- and microbiota-derived products can also influence the brain, for example, through the release of cytokines, hormones such as 5-hydroxytryptamine or stimulation via afferent neural pathways of the vagus nerve and spinal cord. These in-turn can influence the composition of the gut microbiota, either directly, or due to physiological effects on the intestine.

Once the prions enter the CNS they cause extensive neuropathology which is characterised by the activation of microglia and astrocytes, accumulations of PrP^{Sc} and neurodegeneration (Fig. 3). Microglia are the tissue macrophages of the CNS and play important roles in maintaining neuronal homeostasis, synaptic remodelling, the removal of dead and dying cells and as a first line of defence against pathogens (De Lucia *et al.*, 2015, Kranich *et al.*, 2010, Prinz & Priller, 2014, Zhan *et al.*, 2014). A change in microglial status from resting to activated is one

of the first pathological features observed in the CNS during prion disease and occurs long before the development of neuropathology (Vincenti *et al.*, 2016).

This activation is characterised by increased expression of the anti-inflammatory cytokines TGF-β and PGE_2, signalling receptors including Trem2, SiglecF, CD200R, and Fcγ receptors, and the development of a highly branched morphology (Lunnon *et al.*, 2011). These are characteristic of an anti-inflammatory profile such as that exhibited by macrophages following their engulfment of apoptotic cells (Fadok *et al.*, 1998). Thus instead of triggering neurodegeneration, the microglial response during prion disease may play an important neuroprotective or pro-neurogenic role in response to the damage caused by the infection (De Lucia *et al.*, 2015). In support of this hypothesis, prion pathogenesis is accelerated when microglia are unable to sequester apoptotic cell remnants as in MFGE-8-deficient mice (Kranich *et al.*, 2010), and delayed in mice deficient in CD14 (a component of the LPS receptor), where increased microglial activation and enhanced expression of anti-inflammatory cytokines such as IL-10 is observed during the preclinical phase (Sakai *et al.*, 2013). Thus, alterations to this phenotype, through the sensing of bacterial LPS from commensal bacteria, could be sufficient to modify the anti-inflammatory/neuroprotective status of microglia during CNS prion disease towards a more pro-inflammatory disease-exacerbating phenotype.

Exciting data show that the development and function of microglia in the CNS is controlled by the gut microbiota. Microglial maturation is compromised in the brains of germ-free mice and coincides with reduced early responses to LPS or virus infection (Erny *et al.*, 2015). A similar impairment to microglial development and function after treatment of conventionally-housed SPF mice with

broad-spectrum antibiotics (cefotoxin, gentamicin, metrinodazole and vancomycin), revealed that the gut microbiota constitutively maintains the homeostasis of microglia under steady-state conditions (Erny *et al.*, 2015). Similar effects were also observed following a 4 week treatment of conventional mice with broad-spectrum antibiotics (Erny *et al.*, 2015). These data imply that reductions to the complexity or abundance of the microbiota during CNS prion disease, such as prolonged use of broad-spectrum antimicrobial treatments, could affect the activation status of microglia and in doing so impair the development of neuropathology.

Although originally undertaken over 40 years ago to address a separate issue (to define the nature of the scrapie agent), prion disease pathogenesis has been studied in germ-free mice (Lev *et al.*, 1971). Conventional mice and germ-free mice were each injected intracerebrally with the Chandler mouse-adapted scrapie isolate (50 µl of 10% scrapie brain homogenate which had been lyophylized and irradiated at 6 M Rad to eliminate contaminating bacteria and viruses). Survival times were apparently extended in germ-free recipients when compared to conventional mice. On face-value these data appear to support our suggestion above that the impaired status of microglia in germ-free mice (Erny *et al.*, 2015) might impede CNS prion pathogenesis. However, it is uncertain from data presented whether this effect was significant (Lev *et al.*, 1971) (Table 3). Furthermore, a subsequent study by Wade and colleagues did not observe a difference in survival time after intracerebral injection of ME7 scrapie prions into germ-free mice, but a prolongation was observed after intraperitoneal exposure (Wade *et al.*, 1986) (Table 3). The inconsistencies between these studies are difficult to explain. However, it is noteworthy that in the Wade study the effects on

prion pathogenesis in germ-free were compared to those colonized with a restricted, defined Gram-positive bacterial flora comprising of particular species of *Clostridium*, *Bacillus*, *Lactobacillus* and *Bacteroides*. This may have particular importance as the microglia in the brains of germ-free mice colonized with three strains of the altered Schaedler flora (*Bacteroides distasonis,* strain ASF 519; *Lactobacillus salivarius,* strain ASF 361; *Clostridium cluster XIV,* ASF 356) also display an immature phenotype (Erny *et al.*, 2015).

The treatment of hamsters with tetracycline antibiotics (doxycycline, tetracycline or minocycline) increased survival time when administered prior to or at the appearance of clinical signs of prion disease (De Luigi *et al.*, 2008), similarly supporting the hypothesis that microbiota-mediated impairments to microglia status (Erny *et al.*, 2015) might impede CNS prion pathogenesis. In contrast, data from a clinical trial show that the daily treatment of clinical CJD patients with doxycycline did not significantly affect disease progression (Haik *et al.*, 2014). However, it is difficult to determine a direct role for the effect of these antibiotics on the microbiota in these studies as tetracyclines can also inhibit PrP^{Sc} conversion and prevent neurotoxicity in cultured neurons (Tagliavini *et al.*, 2000).

Dietary effects on the microbiota: Many mammalian species rely on constituents of the gut microbiota to break down indigestible dietary components. For example, Bacteroidetes and Clostridia metabolise the polysaccharides in dietary fibre into short chain fatty acids (SCFA). Of these, butyrate plays an important role in regulating inflammation and maintaining the mucosal barrier (Furusawa *et al.*, 2013), and the regulation of microglial homeostasis in the CNS (Erny *et al.*, 2015). The administration of SCFA to germ-free mice restores

microglial development, whereas GPR43-deficient mice, which lack the receptor for these SCFA, have severely malformed microglia (Erny *et al.*, 2015). The "Western diet" is typically high in fat and simple carbohydrates, and can dramatically and rapidly alter the microbiota composition (Turnbaugh *et al.*, 2009), increasing the abundance of Firmicutes, and decreasing the abundance of Bacteroidetes which are important providers of SCFA (Magnusson *et al.*, 2015, Turnbaugh *et al.*, 2009). Thus, Western diet-induced changes to the microbiota could reduce the availability of microbial metabolites such as SCFA, and in doing so, impair microglial development and function, influencing CNS prion disease. Although undertaken for a separate issue, the effects of a high-fat diet on CNS prion pathogenesis have been addressed (Zhu *et al.*, 2015), but no significant effects on prion disease progression, PrP^{Sc} deposition in the brain, astrogliosis or microglial activation were observed.

Although further studies are necessary to reconcile certain discrepancies between studies, the available data suggest that dramatic changes to the abundance or complexity of the commensal gut microbiota have the potential to modify the development and function of microglia, and in doing so, influence CNS prion pathogenesis.

Direct effects of the gut microbiota on prions

The disease-specific isomer of the prion protein, PrP^{Sc}, accumulates in affected tissues in insoluble aggregates that are relatively resistant to proteinase digestion and can transmit disease to recipients (Bolton *et al.*, 1982, Prusiner *et al.*, 1982). When prions are shed into the environment, they can bind to soil particles and can remain infectious for long periods (Bartelt-Hunt & Bartz, 2013). Despite this

resilience ovine alimentary fluids appear to have sufficient proteolytic capacity to digest disease-specific PrP derived from sheep scrapie or BSE (Dagleish *et al.*, 2010, Jeffrey *et al.*, 2006). However, BSE agent-derived PrP^{Sc} can survive incubation with bovine ruminal and colonic microbiota preparations (Bohnlein *et al.*, 2012) or raw sewage (Maluquer de Motes *et al.*, 2012). Whether effects on prion infectivity mirror these observations is uncertain, as bovine ruminal and colonic microbiota preparations can degrade hamster 263K prion-derived PrP^{Sc}, but prion infectivity is retained (Scherbel *et al.*, 2006, Scherbel *et al.*, 2007). While the combined actions of host and microbiota-derived proteolytic enzymes may contribute to the partial digestion of certain prion isolates in the gut lumen, detectable levels of infectious prions can survive these processes and are secreted in the faeces of BSE-, CWD- and scrapie-infected hosts (Kruger *et al.*, 2009, Maluquer de Motes *et al.*, 2008, Safar *et al.*, 2008, Tamguney *et al.*, 2009), and can survive passage through the gastrointestinal tracts of coyotes (*Canis latrans*) (Nichols *et al.*, 2015) and crows (VerCauteren *et al.*, 2012). One study has also proposed that bacterial components, such as LPS, may potentially enhance the abnormal folding of recombinant PrP (Saleem *et al.*, 2014).

Effects of congruent gastrointestinal pathogen infection on prion disease pathogenesis

Effects on CNS prion disease: Congruent infection with a gastrointestinal pathogen and systemic inflammation can each dramatically influence the progression of certain neurodegenerative diseases. For example, systemic inflammation has also been associated with increased cognitive decline in Alzheimer disease (Holmes *et al.*, 2009), and in pregnant mothers can promote

abnormal cortical development in their offspring (Choi *et al.*, 2016). A Th1-polarized systemic immune response following infection with the large intestine-restricted helminth *T. muris* can exacerbate ischemic brain damage in a stroke model (Denes *et al.*, 2010). Conversely, helminth-infections may also have beneficial effects and modulate immune responses and disease severity in multiple sclerosis patients (Correale & Farez, 2013).

The anti-inflammatory cytokine milieu in the brain during prion disease, characterised by elevated expression of TGF-β, acts to regulate the microglial response to minimize CNS inflammation (Cunningham *et al.*, 2002). Consistent with this hypothesis are the demonstrations that CNS prion disease pathogenesis is exacerbated in the absence of typical anti-inflammatory cytokines, including IL-4, IL-10 and IL-13 (Tamguney *et al.*, 2008, Thackray *et al.*, 2004). However, the microglia can switch to a more pro-inflammatory profile upon systemic pathogen infection or exposure to pathogen-associated molecular patterns such as bacterial LPS (Combrinck *et al.*, 2002, Cunningham *et al.*, 2005, Lunnon *et al.*, 2011). These changes to microglial status acutely exaggerate the cognitive decline, impair motor coordination and accelerate CNS prion disease progression (Combrinck *et al.*, 2002, Cunningham *et al.*, 2009, Cunningham *et al.*, 2005).

Effects on prion uptake and neuroinvasion from the lumen of the small intestine

A congruent gastrointestinal pathogen infection may have a wide range of effects on oral prion disease pathogenesis and susceptibility. For example, lesions to the mucosa can enhance the oral transmission of prions (Denkers *et al.*, 2011). Pathogen exposure can also modify the expression of PrP^C and innate immunity

genes in the gut mucosa (Dervishi *et al.*, 2015, Sigurdson *et al.*, 2009). Therefore, gastrointestinal pathogen-mediated damage to the gut mucosa may exacerbate disease pathogenesis by enhancing prion uptake from the lumen. Increased damage to the gut epithelium may also exacerbate the amount of prions shed into the environment (Bessen *et al.*, 2012). Discussed below are the many ways in which a congruent gastrointestinal pathogen infection may affect oral prion disease pathogenesis and susceptibility.

Effects on M cells: The majority of the epithelial cells in the lining of the intestine function to absorb nutrients from the lumen. However, M cells within the epithelia overlying the GALT, are specialized to acquire and transfer particulate microbial antigens across the intestinal epithelium (termed *transcytosis*). The transcytosis of particulate antigens by M cells is important for their delivery to other immune cells in the GALT for the induction of efficient mucosal immune responses (Mabbott *et al.*, 2013). M cells are also important early sites of prion uptake from the intestinal lumen, and oral prion disease susceptibility is reduced in their absence (Donaldson *et al.*, 2012, Heppner *et al.*, 2001, Takakura *et al.*, 2011). Infection with pathogenic microorganisms such as *Salmonella* or stimulation from pathogen-derived effector molecules such as cholera toxin enhance the density of M cells in the intestinal epithelium (Savidge *et al.*, 1991, Tahoun *et al.*, 2012, Terahara *et al.*, 2008). Thus an increased abundance of M cells in the gut epithelium as a consequence of a congruent pathogen infection could enhance the uptake of prions from the gut lumen and increase disease susceptibility.

Effects on mononuclear phagocytes (MNP): After transcytosis into GALT by M cells, the prions are then conveyed to the FDC within the B-cell follicles. The mechanism by which this occurs is uncertain, but the prions are subsequently acquired by MNP (a heterogeneous population of macrophages and classical dendritic cells) in the underlying sub-epithelial dome region (Kujala *et al.*, 2011). These MNP have been proposed to act as "Trojan horses" and shuttle prions towards the FDC (Huang *et al.*, 2002, Mabbott & Bradford, 2015, Raymond *et al.*, 2007). In the steady state the MNP are typically restrained within the lamina propria of the gut (the thin layer of connective tissues immediately beneath the epithelium) (Fig. 4a&b). However, the presence of certain pathogenic microorganisms such as *Salmonella* or *Aspergillus* within the lumen of the small intestine stimulates the recruitment of MNP to the epithelium where they open the tight junctions between epithelial cells and insert their dendrites directly into the lumen to sample the contents (Farache *et al.*, 2013, Niess *et al.*, 2005, Rescigno *et al.*, 2001, Vallon-Eberhard *et al.*, 2006) (Fig. 4c). Thus, the enhanced uptake of lumenal antigens in response to congruent pathogen infection in the small intestine may enhance the uptake of prions from the gut lumen, increasing disease susceptibility.

In the absence of tissue macrophages the accumulation of prions in the Peyer's patches (Maignien *et al.*, 2005) and spleen is enhanced (Beringue *et al.*, 2000), suggesting that some MNP populations play a protective role by degrading prions (Macpherson *et al.*, 2004). Therefore, a significant influx of macrophages into the lamina propria of intestine, as occurs in response to certain pathogen infections (deSchoolmeester *et al.*, 2003, Little *et al.*, 2005) (Fig. 5b), could increase disease susceptibility by enhancing prion sequestration.

Congruent pathogen infection in the large intestine

Although prions do not accumulate within the large intestinal GALT during the early stages of infection (Donaldson *et al.*, 2015b, Gonzalez *et al.*, 2009, Thomsen *et al.*, 2012, van Keulen *et al.*, 2000), pathogen-induced pathology in the large intestine might influence disease susceptibility by enhancing the uptake of prions into GALT within it. Infection with the helminth *T. muris* is entirely restricted to the caecum and proximal colon (Fig. 5). We reasoned that pathology caused by *T. muris* as it burrows within the gut epithelium (Wakelin, 1967) could increase disease susceptibility by increasing the uptake of prions (Fig. 5a). Expulsion of the parasite at the later stages of infection coincides with the influx of large numbers macrophages into the lamina propria of the caecum (deSchoolmeester *et al.*, 2003, Little *et al.*, 2005) (Fig. 5b). This might decrease oral prion disease susceptibility due to the increased sequestration of prions by macrophages (see above). *T. muris* infection also stimulates ILF development in the large intestine (Donaldson *et al.*, 2015b, Little *et al.*, 2005) (Fig. 5c). However, pathogen infection in the large intestine did not significantly affect survival time after oral prion exposure, irrespective of the time at which mice were co-exposed with prions in relation to the *T. muris* infection (Donaldson *et al.*, 2015b) (Table 2).

An independent study has reported that congruent infection with *S.* Typhimurium exacerbated oral prion disease (Sigurdson *et al.*, 2009). In this study the effects on prion disease pathogenesis were attributed to the acute inflammation that infection with the bacterium causes in the colon. However, whereas *T. muris* infection in mice is entirely restricted to the caecum, as discussed above, *S*. Typhimurium infection can affect M cells (Tahoun *et al.*, 2012)

and MNP (Farache *et al.*, 2013, Rescigno *et al.*, 2001, Vallon-Eberhard *et al.*, 2006) in the small intestine which have key roles in oral prion disease pathogenesis. Thus although these data appear to contradict those describing the effects of *T. muris* infection on oral prion disease pathogenesis (Donaldson *et al.*, 2015b), congruent *S*. Typhimurium infection may also have enhanced the uptake of prions from the small intestine.

Changes to the microbiota in other tissues

Mammary gland and mastitis: The lactating mammary gland and breast milk contain complex ecosystems of commensal bacteria including *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* (Jeurink *et al.*, 2013). Chronic inflammatory conditions such as mastitis can induce the extravasation of inflammatory lymphocytes and leukocytes, and the formation of ectopic germinal centres containing FDC in the inflamed tissue (Gommerman & Browning, 2003). The ectopic lymphoid follicles within the mammary glands of sheep with mastitis and coincident scrapie infection can act as ectopic sites of prion replication (Ligios *et al.*, 2005). Furthermore, scrapie-affected sheep with congruent lentiviral mastitis secrete prions into their milk at levels sufficient to transmit disease to suckling lambs (Ligios *et al.*, 2011).

Influence of prion disease on the gut microbiota

At the time of writing we were not aware of any published data describing the effects of prion infection on the gut microbiota. Clinically-affected individuals often display loss of appetite, dehydration, constipation and abnormal faecal transit time during the clinical phase. This could dramatically alter the diversity of the

microbiota in clinically affected individuals. However, whether this influences the progression or onset of clinical signs remains to be determined and will depend on the magnitude and nature of the microbial species affected.

Concluding remarks

In this review we have drawn together data from a wide range of studies, which directly or indirectly, enable us to assess how changes to the gut microbiota could influence prion disease pathogenesis and susceptibility. Although further studies are necessary to resolve certain discrepancies, or address important knowledge gaps, the available data suggest that dramatic changes to the abundance or complexity of the commensal gut microbiota (eg: after prolonged use of antibiotics) have the potential to modify the development and function of microglia, and in doing so, influence CNS prion pathogenesis. Studies have also shown that a congruent pathogen infection or systemic inflammation can dramatically alter prion disease progression and susceptibility. Within the CNS these effects appear to be mediated through the modification of the microglial response to the prion infection. Whereas in the intestine, data suggest that congruent pathogen infection in the small intestine may enhance the uptake of prions from the gut lumen. Pathogen-mediated pathology specifically in the large intestine, in contrast, has little effect on prion pathogenesis.

Following the emergence of BSE in the 1980s estimates suggest more than 500,000 infected cattle may have entered the UK food chain (Valleron *et al.*, 2001, Wilesmith, 1993). Despite this apparent widespread exposure of the UK population to the BSE agent through the food chain the number of confirmed clinical cases of vCJD fortunately remains low (Bishop *et al.*, 2013). However,

data from the retrospective analyses of PrP^{Sc} accumulation in archived appendiceal samples (Gill *et al.*, 2013) suggest that the prevalence of vCJD infection may be much higher. This implies the potential existence of a subclinical carrier state (Clewley *et al.*, 2009, Garske & Ghani, 2010). Understanding how this preclinical phase is maintained will be important to determine the factors which influence the risk of developing clinical prion disease, and the design of novel opportunities for therapeutic intervention. Thus, it will be important to determine whether factors such as dramatic changes to the commensal microbiota (such as use of broad-spectrum antibiotics or dietary changes) or congruent pathogen infection can enhance disease progression in these subclinical individuals.

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Figure legends

Fig. 1. Effects of the microbiota on isolated lymphoid follicle (ILF) development in the intestine. a) ILF abundance is reduced in the small intestines of germ-free mice, whereas their development is induced upon microbial colonisation. This contrasts the situation in the large intestine where the microbiota suppresses ILF development. Tissues were whole-mount immunostained to detect B cell-containing ILF (B220, green) and CD35 (red) to detect FDC-containing mature ILF. White arrowheads, immature ILF; red arrowheads, mature ILF; Bar = 500 μm. b) Total number of ILF in the ileum of the small intestines of germ-free and conventionalised mice. Data adapted from Donaldson et al. (Donaldson *et al.*, 2015a). c) Peyer's patches and mature FDC-containing ILF in the small intestine are important early sites of prion accumulation after oral exposure. Within these tissues, high levels of disease-specific PrP (PrP^{d}, brown) are detected in association with FDC (CD21/35, brown). Treatment of adjacent sections with proteinase K indicates the presence of relatively proteinase-resistant, prion-specific PrP^{Sc} (blue/black). Images adapted from Donaldson et al. (Donaldson *et al.*, 2015b), copyright © American Society for Microbiology, J. Virol. (2015) 89:9532–9547. doi:10.1128/JVI.01544-15.

Fig. 2. A chronic *Trichuris muris* infection in mice dramatically alters the composition of the commensal gut microbiota. (A) Bacterial taxa ploys showing the family level changes in the composition of the microbiota within faeces at intervals up to 35 days after *T. muris* infection. (B) Comparison of the composition of the microbiota in faeces and the caeca of naïve mice and at 35 days after *T. muris* infection. This figure is reproduced from Holm et al. ((Holm *et al.*, 2015);

Fig. 3. Neuropathology in the brains of mice with clinical prion disease. Once the prions enter the CNS the characteristic neuropathology they cause includes neurodegeneration (vacuolation in upper right-hand H&E images) and extensive microglial activation (Iba1, brown, lower panels). Scale bars, 100 μm.

Fig. 4. Mononuclear phagocytes (MNP) in the lamina propria. a) En-face, whole-mount image of the lamina propria in the small intestine of a CSF1R-EGFP mouse. b) In the steady state the MNP are typically restrained within the lamina propria. c) In the presence of pathogenic microorganisms such as *Salmonella* or *Aspergillus*, MNP are recruited to the epithelium where they open the tight junctions between epithelial cells, enabling them to insert their dendrites directly into the lumen to sample the contents (Farache *et al.*, 2013, Niess *et al.*, 2005, Rescigno *et al.*, 2001, Vallon-Eberhard *et al.*, 2006).

Fig. 5. Oral infection of mice with the nematode parasite *Trichuris muris* causes pathology specifically within the caecum. Mice were orally infected with approximately 200 infective *T. muris* eggs and tissues collected at the intervals indicated for further analysis. a) *T. muris* establishes infection in the caecal epithelium. Left-hand panels show autofluorescent immature worms adhered to the caecal epithelium in whole-mount prepared tissue specimens. Arrowheads, ILF ($B220^+$ cells, green). H&E image shows the close association of *T. muris* with

the caecal epithelium/lamina propria (arrows) and sites of damage the parasite causes to the gut epithelium. b) Pieces of ileum and caecum were collected at intervals after *T. muris* exposure and immunostained to detect macrophages ($CD11b^+$ cells, green). *T. muris* infection stimulates the influx of macrophages into the lamina propria of the caecum, but not in the ileum of the small intestine. c) The distal 8 cm of ileum and the entire caecum from control mice, or *T. muris*-
1010 infected mice (28 days post-infection), were whole-mount immunostained to detect isolated lymphoid follicles (individual B cell follicles; $B220^+$ cells; green). This analysis shows that *T. muris* infection stimulates the development of abundant isolated lymphoid follicles (arrows) in caecum. In the ileum, in contrast, the number and density of isolated lymphoid follicles was unchanged after *T. muris* infection. Figure adapted from Donaldson et al. (Donaldson *et al.*, 2015b), copyright © American Society for Microbiology, J. Virol. (2015) 89:9532–9547. doi:10.1128/JVI.01544-15.

1020 **Table 1.** Prion diseases of humans and animals

Prion disease	**Affected Species**	**Route of Transmission**
Iatrogenic Creutzfeldt-Jacob disease	Human	Accidental medical exposure to CJD-contaminated tissues or tissue products
Sporadic Creutzfeldt-Jacob disease	Human	Unknown. Theories include somatic mutation or spontaneous conversion of PrP^{c} to PrP^{Sc}
Variant Creutzfeldt-Jacob disease	Human	Ingestion of BSE-contaminated food or transfusion of blood or blood products from CJD-infected blood donor
Familial Creutzfeldt-Jacob disease	Human	Germ-line mutations of the *PRNP* gene
Gerstmann-Straussler-Scheinker syndrome	Human	Germ-line mutations of the *PRNP* gene
Kuru	Human	Ritualistic cannibalism
Fatal familial insomnia	Human	Germ-line mutations of the *PRNP* gene
Bovine spongiform encephalopathy	Cattle	Ingestion of contaminated food
Scrapie	Sheep, goats, mouflon	Acquired. Ingestion, horizontal transmission, vertical transmission unclear
Chronic wasting disease	Elk, deer, moose	Acquired, ingestion, horizontal transmission, vertical transmission unclear
Transmissible mink encephalopathy	Mink	Acquired (ingestion) source unknown
Feline spongiform encephalopathy	Domestic and zoological cats	Ingestion of BSE-contaminated food
Exotic ungulate encephalopathy	Nyala, kudu,	Ingestion of BSE-contaminated food

Abbreviations used: BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; *PRNP*, the gene that encodes PrP^{c}.

Table 2. Congruent infection with the natural mouse, large intestinal, helminth pathogen *Trichuris muris* does not influence oral prion disease pathogenesis*

Conditions	Day post-*T. muris* infection when mice were orally infected with prions†	Pathological effects of *T. muris*-infection on large intestine	Mean prion disease duration (days±SE)	Disease incidence‡
Prions only	none	none	343 ± 9	8/8
T. muris + prions	0	none	350 ± 7	7/7
T. muris + prions	+7	Damage to gut epithelium	356 ± 12	7/7
T. muris + prions	+21	Influx of intra-epithelial macrophages	356 ± 13	8/8
T. muris + prions	+42	Approx. 7 d after *T. muris* expulsion	347 ± 4	7/7

* Data adapted from Donaldson et al. (Donaldson *et al.*, 2015b).

† Mice were orally-infected with *T. muris* and subsequently orally-exposed to ME7 scrapie prions on the days indicated.

‡ Incidence = no. animals displaying clinical signs of prion disease/no. animals tested.

Table 3. Prion disease in two independent studies using germ-free mice

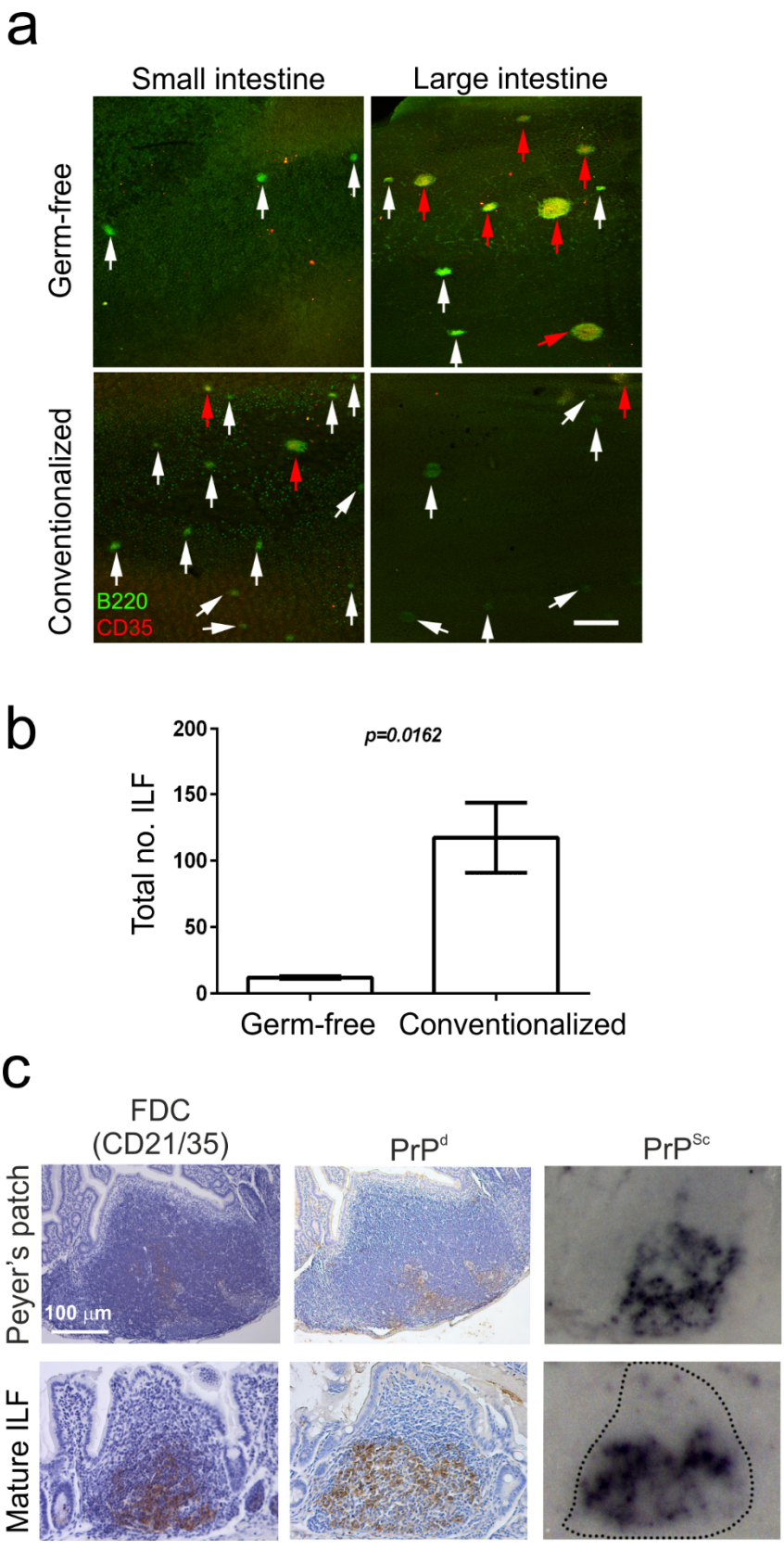
Mouse microbiological status	Route of prion infection	Mean prion disease duration (days)	Disease incidence‡	Study	Reported *P* value
Conventional*	IC	N/A	15/18	(Lev *et al.*, 1971)	
Germ-free	IC	N/A	8/19		>0.05
Defined flora†	IC	151	N/A	(Wade *et al.*, 1986)	
Germ-free	IC	148	N/A		NS
Defined flora	IP	190	N/A		
Germ-free	IP	249	N/A		0.05

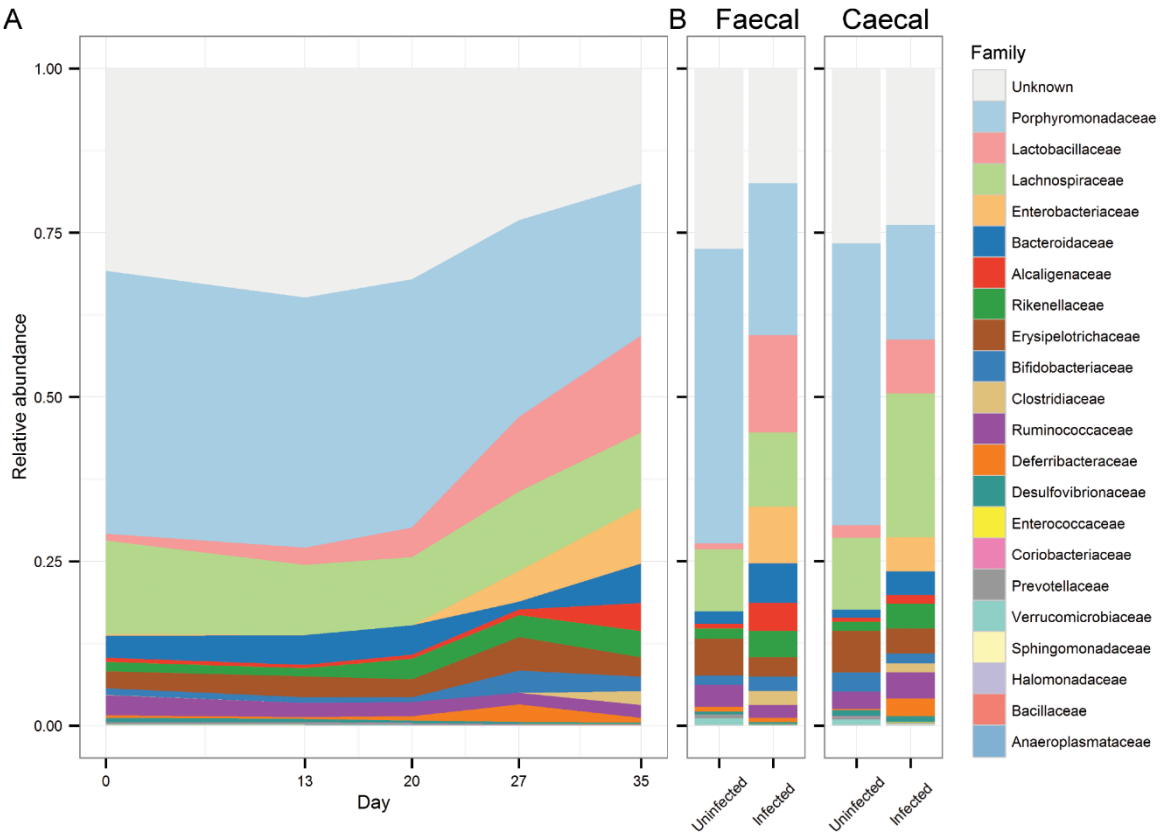
* In the Lev study mice were observed for clinical signs of prion disease up to 23 weeks after exposure (Lev *et al.*, 1971).

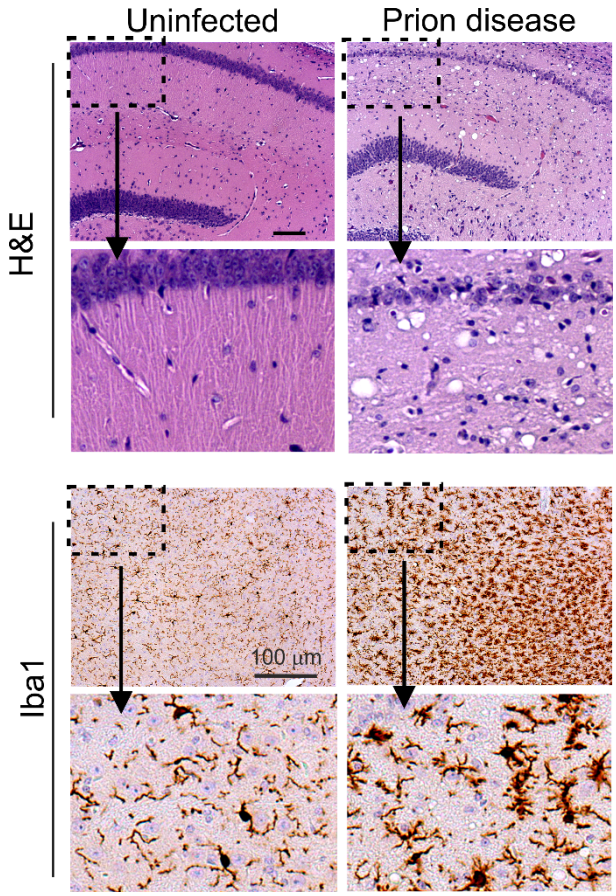
† In the Wade study the effects in germ-free were compared to those colonized with a restricted, defined Gram-positive bacterial flora comprising of particular species of *Clostridium*, *Bacillus*, *Lactobacillus* and *Bacteroides* (Wade *et al.*, 1986).

‡ Incidence = no. animals culled due to clinical signs of prion disease/no. animals tested.

Abbreviations used: IC, intracerebral; IP, intraperitoneal; N/A, insufficient data reported.


a
Small intestine
Large intestine
Germ-free
Conventionalized
B220
CD35
b
p=0.0162
Total no. ILF
200
150
100
50
0
Germ-free
Conventionalized
c
FDC
(CD21/35)
PrP^d
PrP^Sc
Peyer's patch
Mature ILF
100 μm


A
B
Faecal
Caecal
Relative abundance
1.00
0.75
0.50
0.25
0.00
0
13
20
27
35
Day
Uninfected
Infected
Uninfected
Infected
Family
Unknown
Porphyromonadaceae
Lactobacillaceae
Lachnospiraceae
Enterobacteriaceae
Bacteroidaceae
Alcaligenaceae
Rikenellaceae
Erysipelotrichaceae
Bifidobacteriaceae
Clostridiaceae
Ruminococcaceae
Deferribacteraceae
Desulfovibrionaceae
Enterococcaceae
Coriobacteriaceae
Prevotellaceae
Verrucomicrobiaceae
Sphingomonadaceae
Halomonadaceae
Bacillaceae
Anaeroplasmataceae



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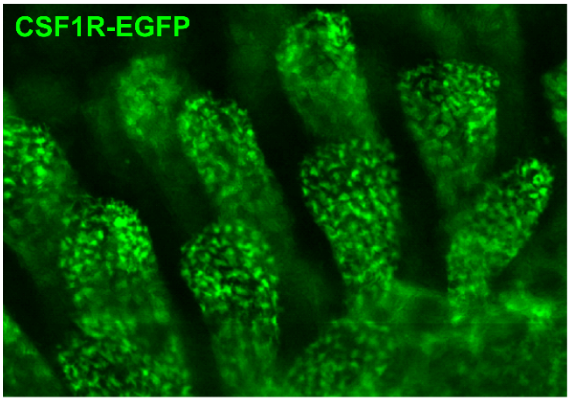
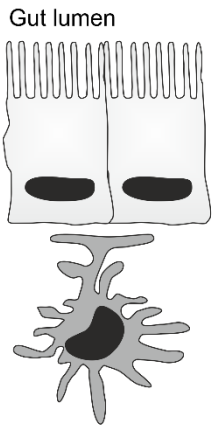
a

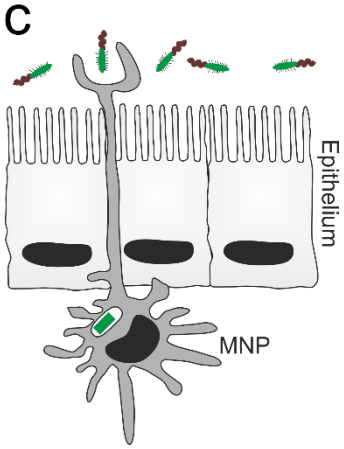


b

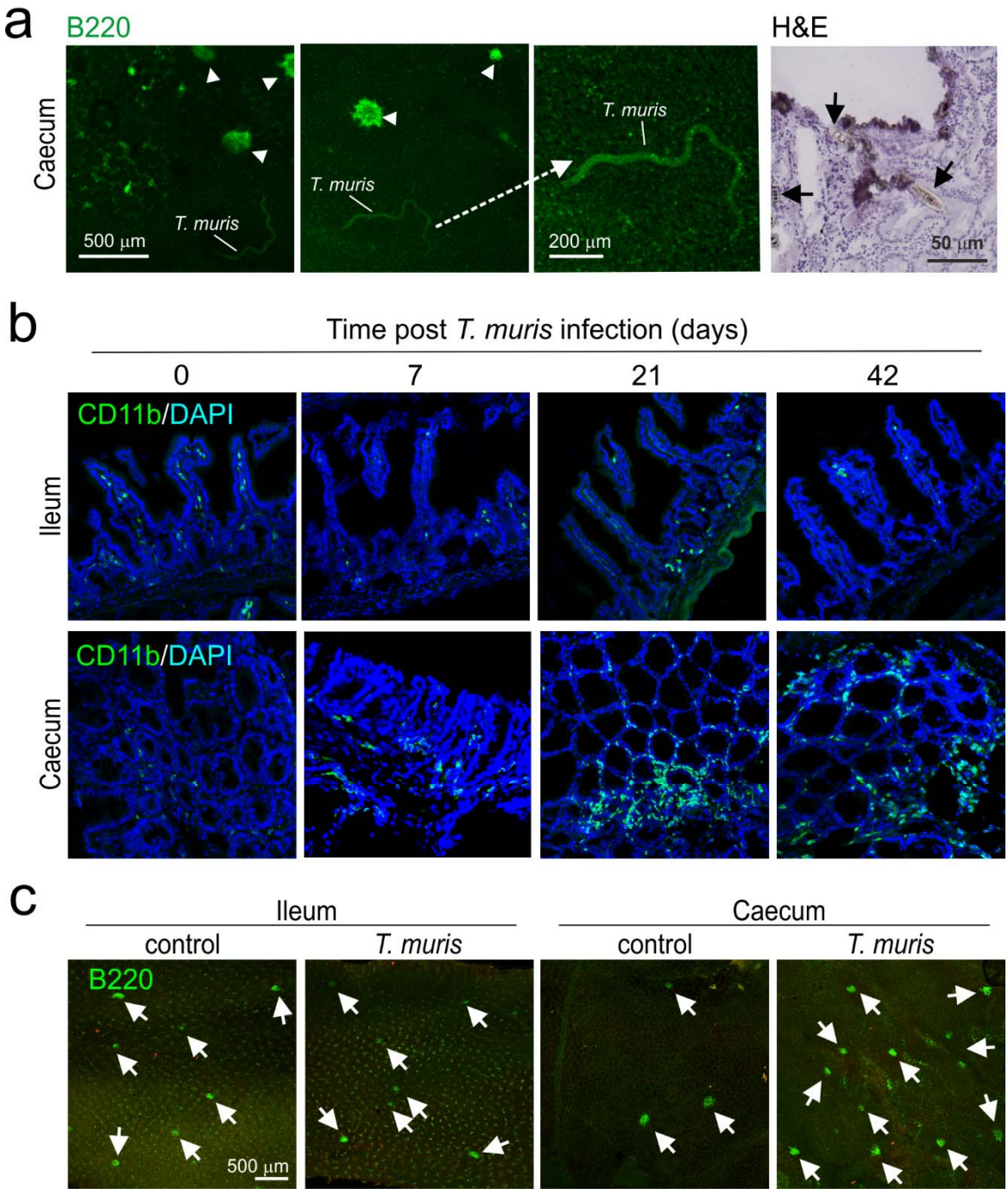


Steady-state

c



Luminal antigen sampling in the presence of pathogenic microorganisms in the lumen


a
B220
H&E
Caecum
T. muris
500 μm
T. muris
T. muris
200 μm
50 μm
b
Time post T. muris infection (days)
0
7
21
42
Ileum
CD11b/DAPI
Caecum
CD11b/DAPI
c
Ileum
Caecum
control
T. muris
control
T. muris
B220
500 μm